ISSN: 0974-5335
IJLST (2016), 9(11):88-95

Effects of D-003 and Lyprinol on acetic acid writhing test in mice

Vivian Molina [1], Zullyt Zamora[2], Licet Mena[3].

1-Titular Researcher, Dr. Pharmaceutical Sciences, Head of the Pharmacology and Toxicology Department,

2-Auxiliar Research, Dr. Health Sciences, Pharmacology and Toxicology Department,

3- Aspiring Researcher, Pharmacology and Toxicology Department,

Centre of Natural Products, National Centre for Scientific Research, Havana, Cuba

vivian.molina@cnic.edu.cu, zullyt.zamora@cnic.edu.cu, licet.mena@cnic.edu.cu

ABSTRACT

Pain is annoying symptom of various acute and chronic inflammatory disorders affecting the quality of life of suffers. Its treatments include anti-inflammatory drugs with adverse effects as gastrointestinal or cardiovascular disorders. Therefore, the search for more secure and effective analgesic drugs is updated. This study investigated the effects of D-003, Lyprinol and the combination (D-003 + Lyprinol) on acetic acid writhing test in mice. Two experimental series were performed. The first evaluated different doses of D-003 (5, 25, 100, 200, 400 and 800 mg/kg) and Lyprinol (5, 25, 100, 200 and 400 mg/kg) on acetic acid-induced writhing in mice, while the second one evaluated the combination therapy D-003 (25 mg/kg) + Lyprinol (25 mg/kg) in this test. Ibuprofen (100 mg/kg) as a reference drug was evaluated too. D-003 (5, 25, 100, 200, 400 and 800 mg/kg) significantly, markedly and dose dependently inhibited the number of writhings in mice compared to the control group (29.3, 33.8, 42, 58.7, 72.9 and 73.7 %, respectively). Lyprinol (25, 100, 200 and 400 mg/kg) produced a significant, modest and non-dose-dependent inhibition of the writhings number compared with the control group (36.3, 35.2, 36.1 and 35.3%, respectively. Ibuprofen (100 mg/kg) marked and significantly inhibited the writhes (56.1%). Comparison of equal doses of D-003 and Lyprinol showed statistically significant differences at the doses of 200 and 400 mg kg, showing greater percent inhibition with D-003 (73%) than with Lyprinol (36%). The combination of D-003+Lyprinol caused a marked inhibition (67.3%) that was statistically significant not only compared with the control group, but with respect to separate D-003 (34.8 %) and Lyprinol (27.9 %), respectively. In conclusion, D-003, Lyprinol and the combined therapy D-003+Lyprinol significantly inhibited acetic acid-induced writhing number in mice, while the combined therapy produced an additional benefit compared to monotherapies with an additive-type positive interaction.

Key words: D-003, Lyprinol, combined therapy, pain, acetic acid-writhing test, mice.

INTRODUCTION

Pain is a distressing symptom that represents a warning about possible damage to organs or tissues, which it has been recognized as one of the main factors currently affecting the quality of life of suffers and therefore its management is one of the biggest challenges for drug therapy [1,2]. The clinical treatment of pain has traditionally included anti-inflammatory drugs (NSAIDs) as the most widely used therapeutic class. However, the use of non-selective NSAIDs has been associated with adverse effects as gastrointestinal disorders that can move from simple dyspeptic symptoms to mucosal ulcerations, which can become complicated with bleeding or perforation being lethal in many cases [3,4], while the use of selective cyclooxygenase type II (COX-2) inhibitors produce cardiovascular disorders such as hypertension and athero-trombosis [5, 6].

On the other hand, the increase in life expectancy in our country and in developed countries with the consequent growth of the elderly population has increased the consumption of NSAIDs [7,8] for relief of pain associated with inflammatory conditions such as osteoarthritis [7], very common disease in these age groups [9].

Therefore, the search for new substances or therapeutic alternatives with analgesic efficacy and better safety profile is a current problem.

Lyprinol, natural extract isolated from algae (*Perna canaliculus*) has been used for the relief of some inflammatory conditions such as osteoarthritis and rheumatoid arthritis, without causing the

ISSN: 0974-5335
IJLST (2016), 9(11):88-95

iatrogenic effects of NSAIDs, whose mechanism of action is based on the dual inhibition of enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) [10, 11].

D-003 is a mixture of higher aliphatic primary acids purified from sugar-cane (*Saccharum officinarum* L.) wax, octacosanoic acid (C28) being the most abundant component, followed by triacontanoic (C30), dotriacontanoic (C32) and tetratriacontanoic (C34) acids. Other acids, such as 1-tetracosanoic (C24), 1-pentacosanoic (C25), hexacosanoic (C26), 1-heptacosanoic (C27), nonacosanoic (C29), tritriacontanoic (C33), hentriacontanoic (C31), pentatriacontanoic (C35) 1-hexatriacontanoic (C36) acids are present at lower concentrations [12].

It has been shown that D-003 has cholesterol-lowering [13-15] and antiplatelet properties [16-18] in experimental and clinical studies, which it has been supported by mechanisms involving the regulation of cholesterol synthesis (HMG-CoA reductase) [19] and inhibition of COX activity, [higher on cyclooxygenase type I (COX-1)], respectively [20].

Moreover, D-003 presents antioxidants [21-23] and beneficial effects on bone (antiresorptive) [24-27] demonstrated in experimental and clinical studies, which together with its efficacy on experimentally induced osteoarthritis in rodents [28, 29] suggests a potential effect to relieve pain (as monotherapy or in combination with other anti-inflammatory alternative) in these types of osteoarticular diseases, where oxidative stress and bone resorption are involved as etiological factors [30, 31].

Experimental toxicology studies have shown no toxicity associated with D-003 treatment [32, 33] while clinical studies have validated its good safety and tolerability [34, 35].

Given the importance of pain as annoying symptom of various acute and chronic inflammatory disorders, the evaluation of potential analgesic effects of D-003, Lyprinol and the combination of them is a topic of interest.

In light of these issues, this study investigated the analgesic effects of D-003, Lyprinol and the combination (D-003 + Lyprinol) in the model of acetic acid-induced writings in mice.

MATERIALS AND METHODS

Animals

Male NMRI mice (20-30g) from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) were used for the study and adapted for 7 days to the following conditions: temperature (22-23^{o}C), relative humidity (55-60%) and 12 hours' dark/light cycles. Food and water were freely supplied.

The experiments were performed in accordance with the care and use of experimental animals prescribed by the Cuban Guidelines for the care of laboratory animals and the Cuban Code of Good Laboratory Practice (GLP), after obtaining the approval of the Institutional Board for animal use.

Administration and dosage

The batch of D-003 (0300010212), supplied by the Plants of Natural Products (National Center for Scientific Research, Havana City, Cuba) was used after corroborate its quality criteria. Batch composition, assessed with a validated specific gas chromatographic method, [36] was as follows: tetracosanoic 0.4%, pentacosanoic 0.3%, hexacosanoic 1.0%, heptacosanoic 3.1%, octacosanoic 57.0%, nonacosanoic 3.0%, triacontanoic 20.0%, hentriacontanoic 1.2%, dotriacontanoic 12.1%, tritriacontanoic 2.0%, tetratriacontanoic 12.0%, pentatriacontanoic 0.5%, hexatriacontanoic 6.1%.

D-003 was prepared as a suspension in Tween 20/H_2O vehicle (2%) and ibuprofen (batch: 111120, Nanjing Baijingyu Pharmaceutical Co, Ltd, Nanjing, China) in a suspension in an acacia gum/water vehicle (1%), while Lyprinol (batch:124441, Blackmores, Australia) was prepared in emulsion form in Tween 65/H_2O (2%).

Two experimental series were performed. The first evaluated different doses of D-003 and Lyprinol on writhing induced by acetic acid in mice in order to determine the appropriate submaximal dose for following evaluation as a combination therapy D-003 + Lyprinol in the second experimental series.

Experimental Series 1

Effects of D-003 and Lyprinol on acetic acid induced writhings in mice.

Mice were randomized into 13 groups (10 mice/group) to which writhing by acetic acid were induced: a control treated with the vehicle, six groups treated with D-003 (5, 25, 100, 200, 400 and 800 mg/kg), five groups treated with Lyprinol (5, 25, 100, 200 and 400 mg/kg), and a reference group with ibuprofen (100 mg/kg).

Experimental Series 2
Effects of combined therapy D-003 + Lyprinol on acetic acid induced writhings in mice.
Mice were randomized into 5 groups (10 mice/group) to which writhings by acetic acid were induced: a control treated with the vehicle, one treated with D-003 (25 mg/kg), one treated with Lyprinol (25 mg/kg), one with combined therapy (D-003 25 mg/kg + Lyprinol 25 mg/kg) and a reference group with ibuprofen (100 mg/kg).

Acetic acid writhings induction in mice.
The mice were fasted 12 hours before the experiment with free access to water. We proceeded according to Lu et al (2007) [37]. One hour after oral administration of single doses of the vehicle, D-003, Lyprinol and ibuprofen each mouse was injected (i.p.) with a solution of acetic acid 2% (0.2 mL/20 g weight). Writhings were then quantified.

Writhings quantification.
The number of writhings produced by each animal during 15 min was quantified 5 minutes post-acetic acid injection. A writhing was defined as a movement consistent stretch in the back arching, stretching the body and extending the hind legs.[39]

Statistical analyses
Comparisons among groups were done with the Kruskal Wallis test; paired comparisons between each treated and control groups with the Mann-Whitney U test. Statistical significance was chosen for $\alpha = 0.05$. Data were processed with the Statistics Software for Windows (Release 6.1 Stat Soft Inc, Tulsa OK, USA). The study of dose-effect relationship was performed by the method of linear regression and correlation using the Primer of Biostatistics (Stanton A Glantz, version 3.01) program. The dose producing 50% of maximum effect or effective dose 50 (ED_{50}) was calculated graphically using the Microcal Origin Version 5.00, Microcal Software, Inc. 1991-1997 program.

RESULTS
Table 1 summarizes the results of the effects of D-003 and Lyprinol on writhing induced by acetic acid in mice. The writhings caused by i.p injection with acetic acid (2%) to mice were inhibited by single oral administration of ibuprofen (100 mg/kg), reference substance, in a marked and significant form (56.1% inhibition).

Oral administration of single doses of D-003 (5, 25, 100, 200, 400 and 800 mg/kg) significantly, markedly and dose dependently ($r=0.94$; $p=0.005$) reduced the number of writhings induced by acetic acid in mice compared to control group, reaching percent inhibition of 29.3, 33.8, 42, 58.7, 72.9 and 73.7, respectively, although slight inhibition achieved with 5 mg/kg was not statistically significant. The ED_{50} was 49.78 mg/kg.

The previous oral treatment with Lyprinol (25, 100, 200 and 400 mg/kg) produced a significant, modest and non-dose-dependent reduction of the number of writhings compared with the control group, with percentages of inhibition of 36.3, 35.2, 36.1 and 35.3, respectively. The dose of 5 mg/kg produced a 20.4% of inhibition but it was not significant.
Comparison of equal doses of D-003 and Lyprinol showed statistically significant differences at the doses of 200 and 400 mg kg, showing greater inhibition percent with D-003 (73%) than Lyprinol (36%).

Table 2 summarizes the effects of combination therapy of D-003 and Lyprinol on writhings induced by acetic acid in mice. The administration with monotherapies of D-003 (25 mg/kg) and Lyprinol (25 mg/kg) produced moderate and significant writhings reduction of 34.8% and 27.9%, respectively, while the combination of both substances caused a marked inhibition of 67.3% that was statistically significant not only compared with the control group but also respect to each separate monotherapy.

ISSN: 0974-5335
IJLST (2016), 9(11):88-95

Table 1. Effects of D-003 and Lyprinol on acetic acid-induced writhing's in mice.

Treatments	Doses (mg/kg)	Writhings (□)	I (%)
Control	0	47.9 ± 3.9	--
D-003	5	33.87 ± 7.12	29.3
D-003	25	31.7 ± 3.8 *	33.8
D-003	100	27.8 ± 4.5 **	42
D-003	200	19.8 ± 2.7***+	58.7
D-003	400	13.0 ± 3.6***+	72.9
D-003	800	12.6 ± 2.35 ***	73.7
Lyprinol	5	38.11 ± 3.57	20.4
Lyprinol	25	30.5 ± 3.8 **	36.3
Lyprinol	100	31.0 ± 3.5 **	35.2
Lyprinol	200	30.6 ± 3.6 *	36.1
Lyprinol	400	31.0 ± 2.1 **	35.3
Ibuprofeno	100	21.0 ± 2.5 ***	56.1

I (%): Percent Inhibition, *p<0,05; ** p<0,01; ***p<0,001 Comparison with control
+ p<0,05; comparison with equivalent doses of Lyprinol (Mann Whitney U test)

Table 2. Effects of the combination therapy of D-003 and Lyprinol on acetic acid-induced writhing's in mice.

Treatment	Doses (mg/kg)	Writhings (□)	I (%)
Control	0	47.7 ± 1.88	--
D-003	25	31.1 ± 2.07 ***	34.8
Lyprinol	25	34.4 ± 3.38 **	27.9
D-003 + Lyprinol	25 + 25	15.6 ± 2.38 ***ab	67.3
Ibuprofen	100	21.0 ± 2.5 ***	56

I (%): Percent Inhibition, ** p<0,01; ***p<0,001 Comparison with control
a p<0,001 comparison with D-003 25 mg/kg; b p<0,001 comparison with Lyprinol 25 mg/kg (Mann Whitney U test)

DISCUSSION

Oral treatment with single doses of D-003, Lyprinol and their combination significantly inhibited the hyperalgesia, measured as the number of writhings induced by intraperitoneal acetic acid injection in mice, while the analgesic efficacy of ibuprofen (reference substance) confirmed the validity of the model in our experimental conditions.

Single oral doses of D-003 (5, 25, 100, 200, 400 and 800 mg/kg) significantly, markedly, and dose-dependently inhibited the number of writhings induced by acetic acid in mice. The minimum effective dose was 25 mg/kg as the lowest dose tested of 5 mg/kg produced a slight inhibition without reaching statistical significance. The maximum effect was observed at the dose of 400 mg/kg because with the highest tested dose of 800 mg/kg a very similar effect was obtained. The ED50 was 49.78 mg/kg.

Lyprinol (5, 25, 100, 200 and 400 mg/kg) produced a moderate, significant and non-dose dependent inhibition of hyperalgesia induced by acetic acid in mice. The minimum effective dose was 25 mg/kg because with the preceding dose of 5 mg/kg there was no-statistically significant inhibition of writhing. The maximum effect was also reached with the same dose of 25 mg/kg, as all subsequent doses tested produced a similar effect, thereby an ED50 was not possible to define.

The comparison between equal doses of D-003 and Lyprinol showed that at doses of 200 and 400 mg/kg D-003 produced higher pain inhibition (73%) than Lyprinol (36%) indicating greater efficacy of D-003. The comparative analysis of the analgesic potency between the two substances have the limitation that the ED50 of Lyprinol was not defined, although both D-003 as Lyprinol have the same minimum effective dose (25 mg/kg), suggesting similar potency.

It has been described that writhings induced by acetic acid in mice is a method for evaluating analgesic substances which act peripherally and/or centrally, although the cause of pain in this model responds generally to the peripheral modulation [39-41].

Thus, acetic acid mainly causes pain by releasing endogenous substances as prostaglandins (PG), serotonin, histamine, bradykinin and substance P, that stimulate local peritoneal receptors involved in this abdominal constriction response [40], being mostly associated with increased levels in peritoneal fluid of prostanoids like PGE2, PGF2α and lipoxygenase products [41]. Central pain modulation occurs via complex processes that involve opiate, serotonergic, dopaminergic and noradrenergic receptors systems [42-44]. In this regard, substances with centrally acting analgesic effects involve this type of receptor system while those who act peripherally inhibit prostaglandins, leukotrienes and/or other endogenous mediators involved in pain.

Recent studies have documented that the mechanism of action of D-003 is based on inhibition of COX activity (greater efficacy over COX-1) [20], as well as a modest inhibitory effect on the activity of the 5-LOX [46]. Since 5-LOX and COX enzymes metabolize arachidonic acid (AA) towards the formation of prostaglandins and leukotrienes, respectively, the analgesic effect of D-003 demonstrated in this study could respond primarily to these peripheral mechanisms involved in pain modulation. However, other possible effects of D-003 on receptors centrally acting should not be discarded and they should be investigated by further studies.

Lyprinol is a dual inhibitor of 5-LOX and COX enzymes [10,11], whose action on the latter includes two isoforms (COX-1/COX-2) [46] and without any reports about affinity preference for any of these (Entrez Pubmed, October 2016).

The analgesic effect of Lyprinol demonstrated in this work could be associated with its effects on these enzymes with consequent reduction of AA metabolites: prostaglandins and leukotrienes. However, the fact that the effect of Lyprinol is moderate and not dose-dependent, unlike the marked and dose-dependent effect of D-003, suggests that COX-1 plays an important role in the development of visceral pain typical of this experimental model.

Likewise, the marked analgesic efficacy of different NSAIDs (aspirin, indomethacin, ibuprofen, diclofenac) reported by various authors in this model [47-50] which is based on the non-selective inhibition of COX, in many cases with a COX-1/COX-2 : 1 ratio, supports this hypothesis.

This result is the first evidence that D-003 is an effective and potent analgesic substance which is a potential benefit to the treatment of pain, although it requires a continuity of studies that allow to characterize its effectiveness in this model with repeated dose regimens as well as in other analgesia models.

On the other hand, this study also demonstrated that single oral administration to mice with the D-003 + Lyprinol combination (25 mg/kg) produced an additional benefit compared to monotherapies, indicating a positive interaction between both substances of additive type, since the efficacy of the combination therapy produced a percentage of inhibition (67.3%) similar to the sum of individual therapies (34.8 and 27.9%).

Taking into account that both D-003 and Lyprinol intervene in the metabolism of AA acting on the 5-LOX and COX enzymes, although with differences in the magnitude of the preferential degree on each of them, at least partially share common mechanisms of action. This may have influenced in the presence of an interaction rather of additive type and not of potentiation or synergism, which requires different mechanisms to take place. However, the demonstration of the additional benefit with the combined therapy is also a potential new alternative for the treatment of pain and further studies should go deeper into this topic.

CONCLUSIONS

Oral treatment with single doses of D-003, Lyprinol and the combined therapy D-003 plus Lyprinol significantly inhibited the number of writhings induced by acetic acid in mice, while the combined therapy produced an additional benefit compared

to monotherapies with an additive-type positive interaction.

REFERENCES

[1] Ruoff G., M. Lema (2003). Strategies in pain management: new and potential indications for COX-2 specific inhibitors. J Pain Symptom Manage, 25(2): S21-S31.

[2] Giovannoni M.P., N. Cesari, A. Graziano, C. Vergelli, C. Biancalani, P. Biagini, V. Dal Piaz (2007). Synthesis of pyrrolo[2,3-d]pyridazinones as potent, subtype selective PDE4 inhibitors. J Enzyme Inhib Med Chem, 22 (3):309-318.

[3] Sinha M., L. Gautam, P.K. Shukla, P. Kaur, S. Sharma, T.P. Singh (2013). Current Perspectives in NSAID-Induced Gastropathy. Mediators of Inflammation, Article ID 258209, http://dx.doi.org/10.1155/2013/258209.

[4] Harirforoosh S., W. Asghar, F. Jamali (2013). Adverse effects of nonsteroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. J Pharm Pharm Sci,16(5):821-847

[5] Nandakishore R., Y. Ravikiran, M. Rajapranathi (2014). Selective cyclooxygenase inhibitors: Current Status. Curr Drug Discov Technol,11(2), 127-132.

[6] Moore R.A., S. Derry, H.J. McQuay (2007). Cyclo-oxygenase-2 selective inhibitors and non-steroidal anti-inflammatory drugs: balancing gastrointestinal and cardiovascular risk. BMC Musculoskelet Disord,8(1):73-77.

[7] Tsumura H., I. Tamura, H. Tanaka, R. Chinzei, T. Ishida, A. Masuda, H. Shiomi, Y. Morita, M. Yoshida, H. Kutsumi, H. Inokuchi, M. Doita, M. Kurosaka, T. Azuma (2007). Prescription of non-steroidal anti-inflammatory drugs and co-prescribed drugs for mucosal protection: analysis of the present status based on questionnaires obtained from orthopaedists in Japan. Internal Medicine, 46 (13): 927-931.

[8] Yajima H., J. Yamao, H. Fukui, Y. Takakura (2007). Up-to-date information on gastric mucosal lesions from long-term NSAID therapy in orthopaedic outpatients: a study using logistic regression analysis. Journal of Orthopaedic Science, 12 (4): 341-346.

[9] Anandacoomarasamy A., L. March (2010). Current Evidence for Osteoarthritis Treatments. Ther Adv Musculoskelet Dis, 2(1): 17–28.

[10] Doggrell S.A. (2009). Lyprinol—is it a useful anti-inflammatory agent? Ecam, doi:10.1093/ecam/nep030, pp. 1-8.

[11] Cho S.H., Y.B. Jung, S.C. Seong, H.B. Park, K.Y. Byun, D.C. Lee, E.K. Song, J.H Son (2003). Clinical efficacy and safety of Lyprinol, a patented extract from New Zealand green-lipped mussel (Perna Canaliculus) in patients with osteoarthritis of the hip and knee: a multicenter 2-month clinical trial. Eur Ann Allergy Clin Immunol, 35(6):212-6.

[12] Más R (2004). D-003: a new substance with promising lipid modifying and pleiotropic effects for atherosclerosis management, Drugs Future, 29: 773–786.

[13] [13] Gámez R., S. Mendoza, R. Más, R. Mesa, G. Castaño, B. Rodríguez, A. García (2000). Dose-dependent cholesterol-lowering effects of D-003 on normocholesterolemic rabbits. Current Therapeutic Research Clinical & Experimental, 61(7): 460-468.

[14] Castaño G., R. Mas, M.L Fernández, J. Illnait, J.C. Fernández, S. Mendoza (2002). Assessment of the effects of D-003, a new antiplatelet and lipid-lowering compound in healthy volunteers: a phase I clinical study. Drugs R & D, 3(5): 337-348.

[15] Castaño G., R. Mas, L. Fernández, J. Illnait, J.C. Fernández, S. Mendoza (2003). Effects of D-003 (5 – 40 mg/day) on lipid profile of patients with Type II hypercholesterolemia: a Phase II clinical study. Clinical Drug Investigation, 23(12): 789-802.

[16] Molina V., M.L. Arruzazabala, D. Carbajal, R. Mas, S. Valdés (2000). Antiplatelet and antithrombotic effects of D003. Pharmacol Res, 42(2):137-143.

[17] Arruzazabala M.L., D. Carbajal, R. Más, V. Molina, G. Castaño (2003). Effects of D-003, a new compound purified from sugar cane wax, on platelet aggregation in healthy volunteers. A randomised double-blind clinical study. Clin Drug Invest, 23(2):107-118.

[18] Arruzazabala M.L, E. López, V. Molina, J. Illnait, D. Carbajal, J.C. Fernández, R. Mas (2010). Efectos del D-003, mezcla de ácidos grados de la caña de azúcar, sobre el perfil lipídico y la agregación plaquetaria de pacientes con diabetes tipo 2. Un ensayo controlado con

placebo. Acta Bioquímica Clínica Latinoamericana, 44(1): 15-24.

[19] Menéndez R., R. Mas, A.M. Amor, I. Rodeiro, R.M. González, S. Jiménez, R. Mas (2001). Inhibition of cholesterol biosynthesis in cultured fibroblasts by D-003, a mixture of very long chain saturated fatty acids. Pharmacology Research, 44(4): 299-304.

[20] Pérez Y., R. Mas, A. Oyarzábal, S. Jiménez, V. Molina (2013). Effects of Policosanol (Sugar Cane Wax Alcohols) and D-003 (Sugarcane Wax Acids) on Cyclooxygenase (Cox) Enzyme Activity In Vitro. Int. J. Pharm. Sci. Rev. Res., 19 (2); 4: 18-23.

[21] Perez Y., R. Menendez, R. Más, R.M Gonzalez, and S. Jiménez (2007). Efecto de al de D-003 producto de la cera de la caña de azucar sobre la peroxidación lipídica in vivo inducida por la administración oral de paracetamol a ratas. Lat. Am. J. Pharm, 26 (1): 57-64.

[22] Pérez Y., R. Mas, R.M. González, S. Jiménez, V. Molina (2008). Effects of D-003 and policosanol on in vivo lipid peroxidation in rats. Arzn-Forsch Drug Res, 58(3):126-130.

[23] Pérez Y., V. Molina, R. Más, R.M. González, S. Jiménez (2008). A comparison of *in vivo* effects of D-003, a mixture of high molecular weight sugarcane wax acids, and grape seed extract on lipid peroxidation markers in rats. Lat. Am. J. Pharm, 27:498-504.

[24] Mendoza S., M. Noa, R. Mas, N. Mendoza (2005). Effects of D-003 (5-200 mg/kg), a mixture of high molecular weight aliphatic acids from sugarcane wax, on bones and bone cell apoptosis in ovariectomized rats. Int. J. Tissue React, 27(4): 213-222.

[25] Noa M., S. Mendoza, R. Mas, N. Mendoza, E. Goicochea (2008). Long term effects of D-003, a mixture of high molecular weight acids from sugarcane wax, on bones of ovariectomized rats: a one-year study. Pharmazie, 63(6): 486-488.

[26] Ceballos A., R. Mas, G. Castano (2005). The effect of D-003 (10 mg/day) on biochemical parameters of bone remodelling in postmenopausal women: a randomized, double-blind study. Int. J. Clin. Pharmacol. Res., 25(4): 175-186.

[27] Ceballos A, G. Castaño, S. Mendoza, J. González, R. Mas, L. Fernández, J. Illnait, M. Mesa, R. Gámez, J.C. Fernández, R. Telles, D. Marrero, M. Gómez, D. Ruiz, Y. Jardines. (2011). Effect of D-003 (10 mg/day) on the bone mineral density of the lumbar spine and femoral neck in postmenopausal women: a randomized, double-blinded study. Korean J. Intern. Med., 26(2):168–178.

[28] Mendoza S., M. Noa, M. Valle, N. Mendoza, R. Mas (2012). Effects of D-003 on formaldehyde-induced osteoarthritis in rats. Int. J. Pharm. Sci. Rev. Res., 16(2), 5: 21-24.

[29] Mendoza S., M. Noa, M. Valle, R. Mas, N. Mendoza (2012). D-003, a mixture of sugarcane wax acids, reduces joint damage in monosodium iodoacetate-induced osteoarthritis in rats. Int J Pharm Sci Rev Res; 16 (2):25-29.

[30] Wang Y., A.M. Hodge, A.E. Wluka, D.R. Englih, G.G. Giles, R. O´Sullivan, A. Forbes, F.M. Cicuttini. (2007). Effect of antioxidants on knee cartilage and bone in healthy, middle-aged subjects: a cross-sectional study. Arthritis Research & Therapy, 9(4): R66.

[31] Abramson S.B., S. Honig (2007) Antiresorptive Agents and Osteoarthritis: More Than a Bone to Pick? Arthritis & rheumatism, 56(8): 2469–2473.

[32] Gamez R., M.D. Rodríguez, C. Alemán, I. Rodeiro, R. Mas, M. Noa, H. García (2007). Toxicología sub-crónica del D-003 en ratones. Lat. Am. J. Pharm, 26: 663- 668.

[33] Gámez R., R. Más, M. Noa, R. Menéndez, H. García, J. González, Y. Pérez, E. Goicochea (2004) Effects of chronic administration of D-003, a mixture of sugar cane wax high molecular weight acids, in beagle dogs. Drugs under Exp and Clin Res, 30(2):75-88.

[34] Castaño G., R. Más, L. Fernandez, J. Illnait, R. Gámez, E. López, J.A. Gutiérrez, J.C. Fernández, E. Alvarez (2002) Assessment of the effects of D-003, a new antiplatelet and lipid-lowering compound in healthy volunteers: a phase I clinical study. Drugs R & D, 3(5):337-348.

[35] Pérez P., J. llnait, L. Femández, M. Mesa, J. Fernandez, R. Gamez, R. Mas, M. Gomez, D. Ruiz, Y. Jardines (2010). Effects of D-003(sugarcane wax acids) on the physical on the static bycicle test. Lat. Am. J. Pharm, 29: 63-270.

[36] Mendez E., D. Marrero, V. Gonzalez, A. Laguna (2003) GC determination of long chain fatty acids that comporse D003 in 5 mg film coated tablets. Journal Pharmacology and Bimolecular Analysis, 31(4): 613-620.

[37] Lu T.C., Y.Z. Ko, H.W. Huang, Y.C Hung, Y.C Lin,

W.H Pengm (2007) Analgesic and anti-inflammatory activities of aqueous extract from Glycine tomentella root in mice. Journal of Ethnopharmacology, 113(1): 1: 142-148.

[38] Kaneria M.S., S.R. Naik, R.K. Kohli (2007) Anti-inflammatory,antiarthritic and analgesic activity of a herbal formulation. Indian J. Experimental Biol, 45(3): 279.

[39] Gené R.M., L. Segura, T. Adzet (1989) Heterothecainuloides: antiinflammatory and analgesic effects. J. Ethnopharmacol, 60(2): 157-162.

[40] Bentley G.A., S.H. Newton, J. Starr (1983) Studies on the Anti-nociceptive Action of Agonist Drugs and their Interaction with Opioid Mechanisms. Br. J. Pharmacol, 79(1): 125-134.

[41] Derardt R., S. Jongney, F. Delevalcee (1980) Release of Prostaglandin E and F in an analgesic Reaction and its Inhibition. Eur. J. Pharmacol, 61(1): 17-24.

[42] Bensreti M.M., R.D. Sewell (1983) Selective effects of dopaminergic modifiers on antinociception produced by different opioid receptor agonists. Pro. Br. Pharmacol. Soc. 6th – 8th July, 70.

[43] Headley P.M., C.T. Shaughnessy (1985) Evidence for opiate and dopamine interaction in striatum. Br. J. Pharmacol, 86: 700.

[44] Wigdor S., G.L. Wilcox (1987) Central and systemic morphine-induced antinociception in mice: of Contribution descending serotonergic and noradrenergic pathways. J. Pharmacol. Exp. Ther, 294(1): 90-95.

[45] Pérez Y., A. Oyarzábal, R. Mas, S. Jiménez, V. Molina (2013) Effect of D-003 on 5-lipooxygenase (5-LOX) enzyme activity in vitro. Rev Cub Farm.; 47(3):400-407.

[46] McPhee S., L.D. Hodges, P.F. Wright, P.M. Wynne, N. Kalafatis, D.W. Harney, T.A. Macrides (2007) Anti-cyclooxygenase effects of lipid extracts from the New Zealand green-lipped mussel, Perna canaliculus. Comp Biochem Physiol B Biochem Mol Biol,146 (3): 346-56.

[47] Shanmugasundaram P., S. Venkataraman (2005) Anti-nociceptive activity of hygrophila auriculata (schum) heine. Afr. J. Trad. CAM, 2 (1): 62- 69.

[48] Florentino I.F., M.V.M. Nascimento, P.M. Galdino, A.F. de Brito, F.F. da Rocha, C.R. Tonussi, T.C. M. de Lima, J.R. de Paula, E.A. Costa (2013) Evaluation of analgesic and anti-inflammatory activities of Hydrocotyle umbellata L., Araliaceae (acariçoba) in mice. An. Acad. Bras. Ciênc, 85(3):987-999.

[49] Mishra D, G.Ghosh, P.S Kumar and P.K Panda (2011) An experimental study of analgesic activity of selective cox-2 inhibitor with conventional NSAIDs. Asian Journal of Pharmaceutical and Clinical Research, 4 (1): 78-81.

[50] Lichtman A.H, P.S Naidu, L. Booker, D.L Boger, B.F Cravatt (2008) Targeting FAAH and COX to Treat Visceral Pain. The FASEB Journal, 22(1):1125.12.